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Continuous synthesis of aminophenols from nitroaromatic compounds by combination of metal and biocatalyst†

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The combined action of immobilized hydroxylaminobenzene mutase and zinc in a flow-through system catalyzes the conversion of nitroaromatic compounds to the corresponding *ortho*-aminophenols, including a novel analog of chloramphenicol.

Aminophenols are important precursors for the synthesis of high performance polymers and biologically active compounds but applications are limited by the complexity and low yields of synthetic routes.[1] With nitrobenzene as a model substrate we describe a continuous reaction system that consists of an initial reduction of nitrobenzene to hydroxylaminobenzene using zinc, followed by enzymatic conversion to *ortho*-aminophenol.

In *Pseudomonas pseudoalcaligenes* JS45, nitrobenzene is reduced to hydroxylaminobenzene (HAB) by a nitrobenzene reductase enzyme. A second enzyme, HAB mutase (EC 5.4.4.1), then catalyzes the rearrangement of HAB to *ortho*-aminophenol which is biodegraded to support growth of the bacteria.[2] *Pseudomonas pseudoalcaligenes* JS45 encodes two distinct enzymes, HAB mutase A and HAB mutase B.[2b] The activity of nitrobenzene reductase and HAB mutase in concert can be exploited to catalyze the conversion of a range of nitroaromatic compounds to yield novel *ortho*-aminophenols,[1b,c] but the use of whole cell systems is limited by product toxicity, poor substrate uptake rates and the substrate specificity of the enzymes. The use of nitrobenzene nitroreductase is limited by the requirement for the cofactor, NADPH. In addition, it is difficult to recover enzymes following catalysis.

Nitroaromatic compounds can alternatively be reduced to the corresponding hydroxylamines by zinc.[3] The hydroxylamine can then be enzymatically rearranged to *ortho*-aminophenol by the HAB mutase.[2b] The activities of enzymes and metal catalysts are often optimal under dissimilar conditions, which limits their use in a single reaction system. The use of combined metal catalysts and biocatalysts however, has recently started to receive attention and a few reports demonstrate the applicability of the approach.[4]

We recently developed a method of enzyme immobilization in biomimetically-derived silica (biosilica) that increases the mechanical stability of the immobilized enzyme and facilitates their application in flow-through reactor systems.[5] Here we immobilized HAB mutase B in biosilica by the same method.[5] A column containing immobilized mutase enzyme was connected in series to a column containing zinc (Fig. 1). When an aqueous solution of nitrobenzene (1 mM) was pumped through the two columns at a flow rate of 0.25 ml min^{-1}, *ortho*-aminophenol (0.89 ± 0.095 mM) was produced continuously for over 5 h (conversion efficiency of 89 ± 1.45%). Small quantities of HAB (66 ± 22 μM) and aniline (97 ± 13 μM) (byproduct of the initial zinc reaction) were detected throughout (Fig. 1). When the flow rate and substrate concentration were increased to 0.5 ml min^{-1} and 5 mM, 3.56 mM (±0.24) *ortho*-aminophenol was produced for over 8 h with no loss in activity indicating a conversion efficiency of 71.2% (±5.83). HAB (0.89 ± 0.31 mM), aniline (0.37 ± 0.07 mM) and nitrosobenzene (0.25 ± 0.024 mM) were detected in the effluent, which suggested that the capacity of the mutase column was exceeded at the higher flow rate.

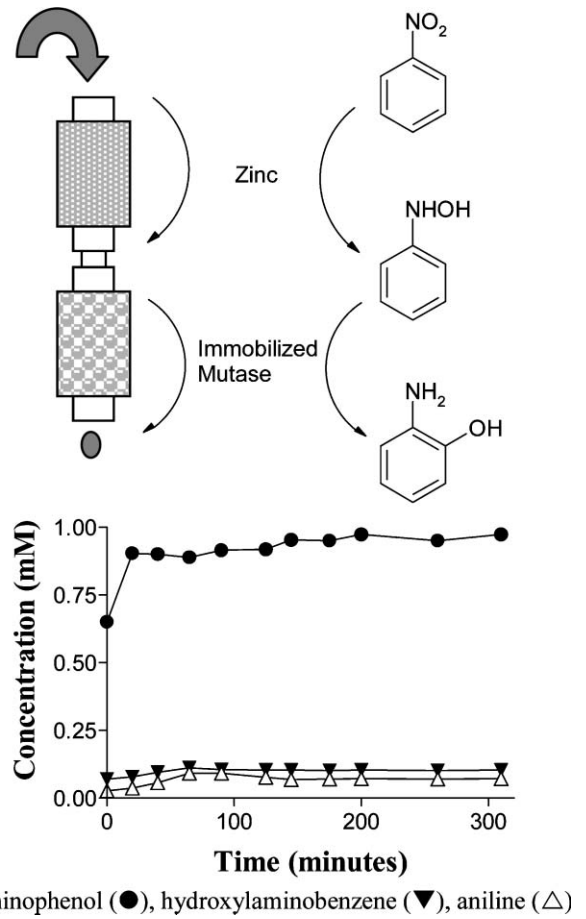

Fig. 1 Transformation of nitrobenzene (1 mM) to *ortho*-aminophenol by a sequential zinc-reduction and mutase-catalyzed reaction system.

† Electronic Supplementary Information (ESI) available: S1: Purification stategy for mutase enzymes HabA and HabB; S2: ^{1}H and ^{13}C NMR structural data for aminophenol analog of chloramphenicol. See http://www.rsc.org/suppdata/cc/b4/b413519a/

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When we investigated the efficiency of the mutase column alone with a feed of 1 mM HAB, the substrate was unstable due to auto-oxidation, which made quantification of the conversion efficiency difficult. One of the advantages to a continuous reaction system is the rapid conversion of the unstable HAB intermediate into *ortho*-aminophenol.

The biosynthesis of antibiotics using intact bacterial cells is inherently limited due to the biocidal properties of the product. An immobilized enzyme reaction system therefore provides an attractive alternative. The use of the zinc/mutase cascade was therefore investigated with the antibiotic chloramphenicol (*N*-[2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl) ethyl]-2,2-dichloro-acetamide) as a model system (Fig. 2). Analogs of chloramphenicol that lack the nitro substituent have been investigated,[6] but the formation of an aminophenol analog has not been reported.

Preliminary investigation demonstrated that the nitro group of chloramphenicol was reduced to a hydroxylamino derivative by reaction with zinc and the identity of the product was confirmed by LC–MS analysis (data not shown). When the hydroxylamino derivative of chloramphenicol was incubated with partially purified HAB mutase B, the product was not converted to the expected aminophenol. HAB mutase A, however, converted the hydroxylamino derivative to the corresponding aminophenol (*N*-[2-(4-amino-3-hydroxy-phenyl)-2-hydroxy-1-hydroxy methyl-ethyl]-2, 2-dichloro-acetamide). This was the first observation of a difference in substrate specificity between the two enzymes. The aminophenol was purified from this reaction by HPLC and characterised by LC–MS (molecular ion, *m*/*z* 308) and NMR.† The success of the batch reaction with chloramphenicol led us to investigate the synthesis of the product using the continuous zinc/mutase column system as described above for the nitrobenzene model system. When an aqueous solution of chloramphenicol (1 mM) was pumped through the two columns at a flow rate of 0.25 ml min^{-1}, the corresponding aminophenol analog

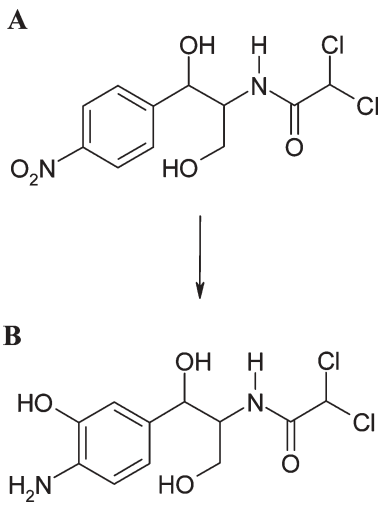

Fig. 2 Structure of chloramphenicol (A) and an aminophenol analog (B).

(1.04 ± 0.029 mM) was obtained continuously for 24 h, demonstrating 100% conversion efficiency and a product formation rate of 0.24 mg h^{-1} mg^{-1} total protein. The zinc becomes oxidized over time. When the column was repacked with new zinc, the system could be continued for a further 24 h.

The flow-through system described can be applied to the transformation of a wide variety of nitroarene substrates into the corresponding aminophenolic products while bypassing many of the current limitations of whole cell biocatalysis. The transformation of antibiotics with nitro functional groups to the corresponding aminophenols may provide a simple method for synthesising novel antibiotic analogs. The high efficiency and regiospecificity of the reported system provides an attractive alternative to conventional chemical synthesis. In addition, the immobilized enzyme can be recovered and subsequently reused.‡

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Notes and references

‡ Biosilica immobilization of partially purified mutase enzyme (†) was performed as described previously.[5] Mutase enzyme activity was determined as described previously.[2b] For continuous flow experiments, columns (XK-16/20, Pharmacia Biotech) were packed with (a) zinc (5 g, 40 mesh) (total 1 ml volume), (b) immobilized mutase from a 5 ml reaction mixture (containing approx. 10 mg protein) and 5 g of glass beads (60/80 mesh, Alltech) (total 10 ml volume). Substrate was pumped through the system at a fixed flow rate and the eluate collected for analysis. The entire apparatus was maintained at 30 °C. Substrates were dissolved in water containing NH_4Cl (40 mM) and sparged with argon to ensure an anaerobic environment. The pH of the reaction remained at approximately pH 7.4 throughout. Reactants and products of nitrobenzene conversion were monitored by reverse-phase HPLC on a Supelco ABZ column with an acetonitrile/water (+0.1% trifluoroacetic acid) gradient. Reactants and products of chloramphenicol transformation were resolved by ion pair chromatography on a Luna C18 column (150 × 2 mm, Phenomenex) with an acetonitrile/triethylamine (10 mM, adjusted to pH 5 with acetic acid) gradient.

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